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Basal expression of copper transporter 1 in intestinal epithelial cells is regulated by hypoxia-inducible factor 2α

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ABSTRACT

Hypoxia, via stabilization of HIF2α, regulates the expression of the intestinal iron transporters DMT1 and ferroportin. Here we investigated whether the intestinal copper importer Ctr1 was also regulated by hypoxia. Copper uptake and Ctr1 mRNA expression were significantly increased in Caco-2 cells exposed to hypoxia. To determine whether HIF2α was involved in regulation of Ctr1 expression, we employed three models of HIF2α knockdown (chemical suppression of HIF2α translation in Caco-2 cells; HIF2α-siRNA-treated HuTu80 cells; HIF2α-intestinal knockout mice); Ctr1 mRNA expression was decreased in all three models under normoxic conditions. HIF2α translational inhibitor did not alter Ctr1 expression under hypoxic conditions. We conclude that basal expression of Ctr1 is regulated by HIF2α; however, the induction by hypoxia is a HIF2α-independent event.

1. Introduction

Iron and copper are essential trace elements in the human diet. There are a number of similarities between the metabolic roles of these metals in the body; for example, both function as catalytic centers in enzymes participating in oxidation–reduction reactions [1–3]. Absorption of both iron and copper is proton-coupled and takes place in the proximal duodenum; prior to absorption, both metals must be present in their reduced form; Cu^{+} and Fe^{2+}, respectively. We have recently shown that duodenal cytochrome *b* (Dcytb), originally identified as a ferric reductase [4] is also a cupric reductase in vitro [5] suggesting that this enzyme may serve a dual function in the intestine and provide a link between iron and copper absorption. Once reduced Cu^{+} and Fe^{2+} are substrates for their respective apical membrane transport proteins, copper transporter 1 (Ctr1; [6,7]) and the divalent metal transporter 1 (DMT1; [8,9]).

There is abundant evidence that changes in either copper or iron status can influence the metabolism of the other metal. The most widely studied link between copper and iron metabolism is ceruloplasmin; a copper-dependent serum ferroxidase that is essential for iron efflux from hepatocytes into the circulation [10]. More recently, the intestinal ferroxidase hephaestin (also a copper-dependent enzyme) has been shown to be essential for iron release from enterocytes [11]. At the apical membrane of intestinal epithelial cells, we [12,13] and others [14] have demonstrated that copper can influence iron uptake via DMT1. Furthermore, there is evidence that iron efflux via ferroportin (FPN) may also be directly influenced by copper status [13,15]. Studies in copper-deficient animals have provided further evidence, at the molecular level, of the impact of copper on iron homeostasis [16–20].

Emerging data indicate that changes in iron status can affect copper transport across the intestinal epithelium [21]. Expression of the intestinal copper efflux transporter ATP7A is increased in iron deficiency [22,23] and furthermore, increased copper levels have been observed in the intestinal mucosa and liver of iron deficient animals suggesting an increase in copper uptake [23,24].

Recent studies in mice deficient in hypoxia inducible factors (HIF) have elucidated potential mechanisms mediating the effects of iron deficiency on intestinal iron transporter expression. HIF2α is stabilized in duodenum in iron deficiency and this transcription factor has been shown to directly regulate the expression of DMT1 and Dcyt*b* [25,26]; and more recently FPN [27]. Interestingly, a recent study in rat IEC6 cells suggests that the copper transporter ATP7A is also regulated by HIF2α [28], providing further evidence

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for the involvement of common pathways in the regulation of iron and copper homeostasis.

Despite a wealth of data indicating that copper regulates iron absorption via DMT1, little is known regarding the regulation of the apical copper transporter Ctr1 in situations where iron homeostasis is impaired. Here, we have explored the possibility that hypoxia, an important modulator of iron metabolism, also influences Ctr1 expression. The potential role of HIF2α as a mediator of these events has also been studied.

2. Materials and methods

2.1. Cell culture and animals

Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), for 21 days. To induce hypoxia, cells were placed in a sealed Perspex chamber. The chamber was then flushed with air containing 1% O_2, 5% CO_2 and 94% N_2; sealed and incubated at 37 °C for 24 h. To investigate the role of hypoxia-inducible factors (HIF1α and HIF2α) in regulating copper transporter expression, cells were incubated for 24 h with either N,N′-(2,5-dichlorosulfonyl) cystamine (20 μM), which suppresses cellular HIF1α levels [29], or Methyl-3-(2-(cyano(methylsulfonyl)methylene)hydrazino)thiophene-2-carboxylate (10 μM), which suppresses the translation of HIF-2α mRNA [30]. Both compounds were used at their apparent IC_{50} concentrations based on previous studies [29,30].

HuTu80 cells were grown and cultured in DMEM containing 10% FBS. Cells were seeded in 12 well-plates to give approximately 50% confluence 24 h after plating and were transfected with 10 nM of either the siCONTROL non-targeting, non-homologous siRNA, (AM 4635) or siRNA targeting Hif-2α (S4698), (Ambion Universal Library, Inc., Lafayette, CO, USA) using Lipofectamine RNAi Max (Invitrogen, Paisley, UK) according to manufacturer's protocol. Transfected cells were harvested after 72 h for RNA extraction and cDNA synthesis as described below.

HIF2α intestinal knock-out mice were generated and maintained as described previously [25].

2.2. Quantitative PCR

Total RNA was isolated using Trizol reagent (Invitrogen) according to manufacturer's instructions. Following first strand cDNA synthesis, expression levels of Ctr1, 18S (housekeeper gene in cultured cells) and cyclophilin (housekeeper gene in mouse duodenum) were analyzed by Real Time PCR using Quanti-Tect SYBR Green PCR kit (Qiagen Ltd., West Sussex, UK) according to manufacturer's protocol (see Supplementary Table 1 for the primer sequences used in PCR reactions). Gene quantification is expressed relative to a housekeeper gene (18S for Caco-2 and HuTu80 cells; cyclophilin for mouse studies) and data have been normalized to the control group in each study.

2.3. Western blotting

Caco-2 cells were scraped into ice-cold phosphate buffered saline (PBS) containing 0.1% SDS and protease inhibitor cocktail (Sigma–Aldrich, IL, USA, 10 μl/ml buffer) and homogenized by passing through a 25-gauge needle several times. Proteins (40 μg/well) were separated by SDS-PAGE. Following immobilization on nitrocellulose, the proteins were exposed to commercially available antibodies against Ctr1 (1:1000 dilution, Novus Biologicals, Cambridge, UK), DMT1 (NRAMP24A, which recognizes all DMT1 isoforms; 1:1000 dilution, Alpha Diagnostics Inc., San Antonio, TX), hypoxia-inducible factors (HIF-1α and HIF-2 α, both at 1:1000 dilution, Novus Biologicals). Glucose Transporter 1 (GLUT1; 1:1000 dilution; a gift from Professor Steven Baldwin, University of Leeds, UK). Immunoreactivity was observed using an HRP-linked secondary antibody (Dako, Cambridgeshire, UK) and ECL Plus (GE Healthcare, Buckinghamshire UK). Actin protein levels in the membrane samples were also measured and acted as a housekeeping control (anti-actin antibody, 1:2000 dilution, Sigma–Aldrich).

2.4. Copper uptake

Copper uptake in Caco-2 cells was measured using inductively couple plasma optical emission spectroscopy (ICP-OES; iCAP 6000 series, Thermo Fisher Scientific, Epsom, UK). Cells were incubated in serum-free DMEM containing 50 μM $CuCl_2$ for 2 h at 37 °C. At the end of the incubation period cells were washed twice with ice cold PBS and harvested into concentrated 68% nitric acid (Thermo Fisher Scientific) and were digested at 90 °C for 90 min. to extract minerals. Cellular copper content was assessed against a standard curve set using a multi-element standard (Merck, Poole, UK) containing 1006 ± 10 mg/l copper in diluted nitric acid.

2.5. Statistical analysis

Data are presented as mean ± S.E.M. Statistical differences ($P \leqslant 0.05$) between groups were determined using either Student's unpaired t-test or a one-way analysis of variance followed by Dunnett's post hoc test where appropriate (SPSS Statistical Package, SPSS UK Ltd., Surrey, UK).

3. Results

3.1. Induction of hypoxia in Caco-2 cells

To study the effects of hypoxia (an important modulator of iron metabolism) on copper uptake and transporter expression, Caco-2 cells were cultured for 24 h in an atmosphere of 1% O_2. Expression of the hypoxia-sensitive proteins, DMT1, GLUT1, and HIF2α were increased following this procedure, confirming the induction of cellular hypoxia (Fig. 1). HIF1α protein was only detectable at very low levels in Caco-2 cells and there was no measurable increase in hypoxic cells. There was a modest increase (approximately 25%) in whole cell Ctr1 protein levels in cells grown under hypoxic conditions.

3.2. Copper uptake

Cellular uptake of copper was measured using ICP-OES. Copper uptake by hypoxic cells was fivefold greater than uptake in control cells grown under normoxic conditions (Fig. 2).

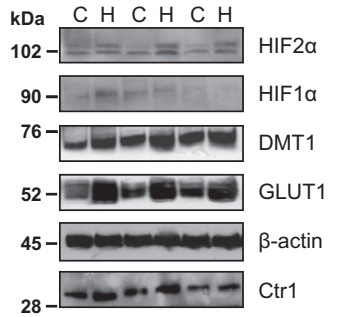

Fig. 1. Expression of hypoxia-induced proteins in Caco-2 cells. Protein levels of the apical copper transporter Ctr1, the iron transporter DMT1, the glucose transporter GLUT1 (a classical hypoxia regulated gene) and the hypoxia inducible transcription factors HIF1α and HIF2α were measured by Western blotting. Representative blots from three normoxic and three hypoxic Caco-2 cell cultures (from a total of 6–9 preparations) are presented together with levels of the housekeeper protein actin.

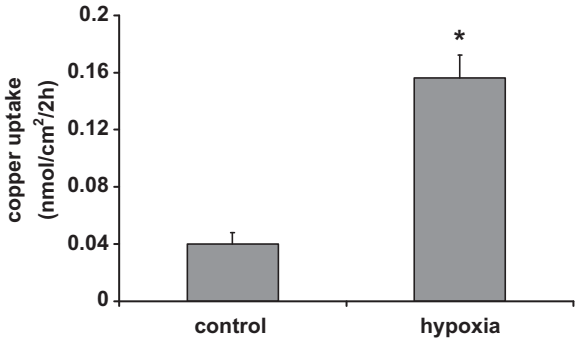

Fig. 2. Copper uptake in Caco-2 cells. Copper uptake was measured in hypoxic Caco-2 cells following exposure to copper (50 μM) for 2 h using ICP-OES. Data are presented as means ± S.E.M. of six experiments in each group. *$P < 0.01$ (Student's unpaired t-test).

3.3. Hypoxia and Ctr1 expression

Increased copper uptake by hypoxic Caco-2 cells was associated with an increase in Ctr1 mRNA expression (Fig. 3A). Since many of the cellular effects of hypoxia are mediated directly at the level of gene transcription by the hypoxia inducible factors HIF1α and HIF2α, we determined whether the expression of the apical copper importer, Ctr1, might also be HIF-regulated. Caco-2 cells grown under normoxic conditions and were exposed to chemical inhibitors of either HIF1α or HIF2α translation. The expression of Ctr1 mRNA was significantly decreased in the presence of the HIF2α inhibitor but was not altered following exposure to the HIF1α inhibitor (Fig. 3B). There was no significant effect of either inhibitor on whole cell Ctr1 protein levels (data not shown).

Recognizing that the chemical inhibitor of HIF2α translation may lack some specificity we sort to further confirm the role of HIF2α in the regulation of Ctr1 expression by using siRNA oligonucleotides to knock down HIF2α in the human duodenal cell line HuTu80. HIF2α mRNA expression was significantly decreased in HIF2α-silenced cells confirming the specificity of the siRNA treatment (Supplementary Fig. 1). Ctr1 expression was also significantly decreased in HIF2α-siRNA treated cells compared with cells transfected with siCONTROL non-targeting, non-homologous siRNA (Fig. 4A). Furthermore, duodenal Ctr1 mRNA expression was significantly decreased in normoxic mice containing intestinal-specific deletion of HIF2α (Fig. 4B).

To ascertain whether HIF2α, in addition to its role in regulating basal Ctr1 expression, also mediated the effects of hypoxia on Ctr1 expression, Caco-2 cells were grown under hypoxic conditions in the presence or absence of HIF chemical inhibitors. However, in contrast to studies in normoxic conditions, there was no effect of the HIF2α translational inhibitor on the hypoxic-induction of Ctr1 mRNA (Fig. 3C).

4. Discussion

The intimate relationship between copper and iron homeostasis has been known for many years (reviewed in [1,3]). Recently the molecular basis for copper–iron interactions at the intestinal level has become clearer. Copper deficiency or copper loading can influence the expression and function of a number of key elements of the intestinal iron transport pathway [13,16–20]. In addition, there is evidence that iron status alters copper absorption and the expression of the copper transporter ATP7A [22–24].

Hypoxia increases intestinal iron absorption in vivo [31,32] and enhances the expression of components of the duodenal iron transport pathway [4,33]. The effects of hypoxia on intestinal copper transport and transporter expression are less clear. Here were showed that copper accumulation by hypoxic cells was fivefold greater than uptake into normoxic control cells. This is the first

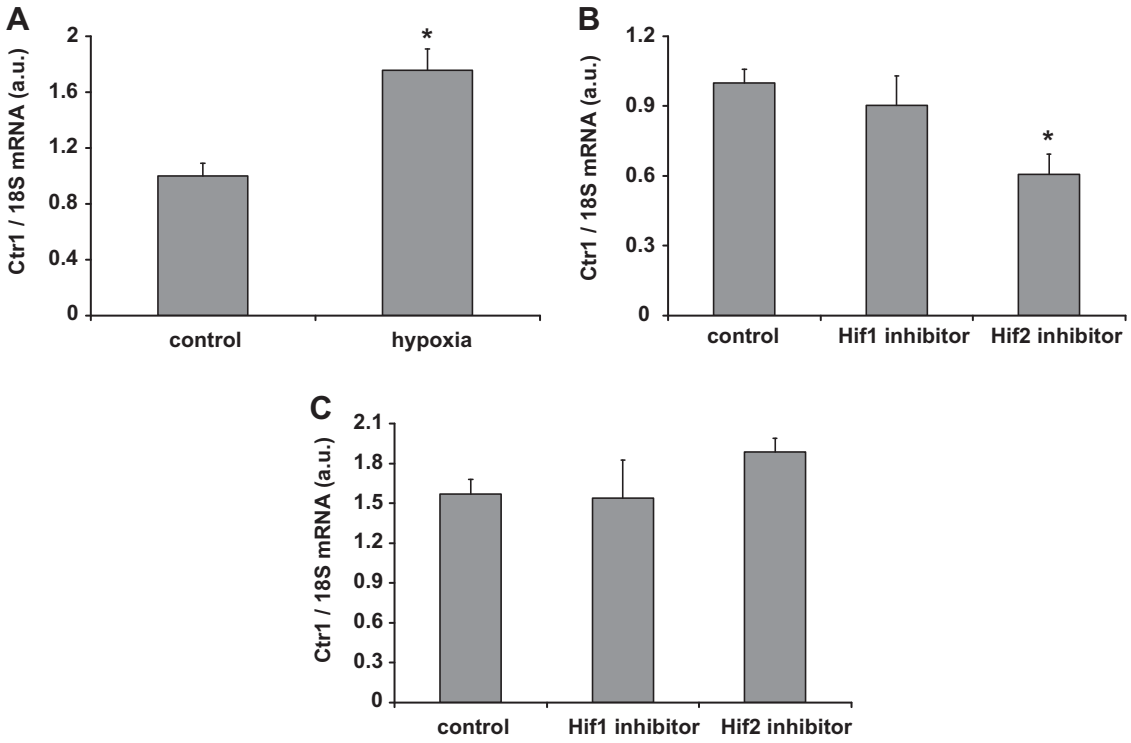

Fig. 3. Regulation of Ctr1 expression in Caco-2 cells. Ctr1 mRNA levels, expressed relative to 18S housekeeper gene (A), were measure by Q-PCR in normoxic and hypoxic Caco-2 cells. Data are presented as means ± S.E.M. of nine observations in each group. *$P < 0.01$. Ctr1 mRNA levels in Caco-2 cells grown under normoxic (B) or hypoxic (C) conditions and exposed to inhibitors of HIF1α (N,N′-(2,5-dichlorosulfonyl) cystamine; 20 μM) or HIF2α (methyl-3-(2-(cyano(methylsulfonyl)methylene)hydrazino) thiophene-2-carboxylate; 10 μM) protein translation ($n = 6$ in each group). *$P < 0.05$ compared with the control group (ANOVA and Dunnett's post hoc test).

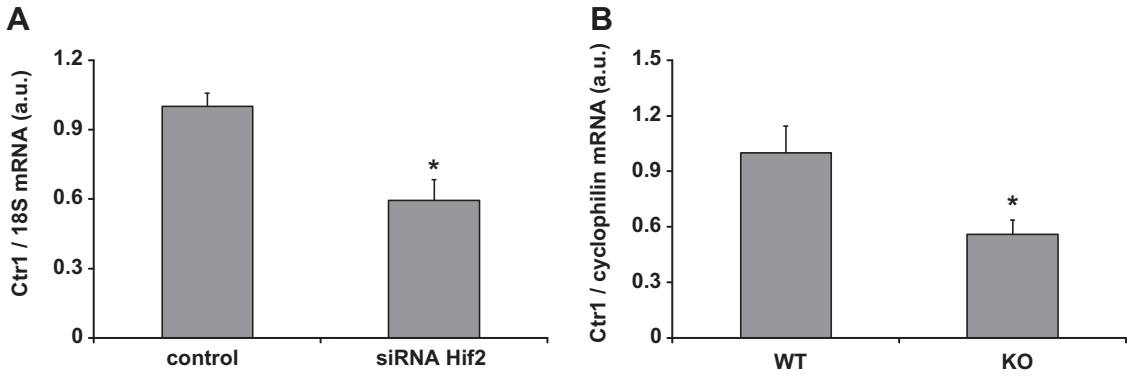

Fig. 4. Ctr1 expression in HIF2α-depleted cells. Ctr1 mRNA levels were measured in HIF2α-siRNA treated HuTu80 duodenal epithelial cells (A) and in duodenal enterocytes (B) isolated from HIF2α intestinal-specific knockout mice (KO) and their wild-type (WT) littermates ($n = 3$ in each group). Data are presented as means ± S.E.M. $^{*}P < 0.05$ (Student's unpaired *t*-test).

demonstration that hypoxia increases intestinal copper absorption and is consistent with previous findings in murine RAW264.7 macrophages [34].

Ctr1 has recently been confirmed as the major apical membrane transporter responsible for the assimilation of copper from the diet [7,35]. Our data show that Ctr1 expression in intestinal Caco-2 cells was increased following induction of hypoxia. Recent studies have shown that the copper efflux transporter ATP7A is also up-regulated in hypoxia in rat intestinal IEC6 cells [28] suggesting that hypoxia may be a common regulator of intestinal iron and copper homeostasis.

Many of the cellular effects of hypoxia are mediated directly at the level of gene transcription by the hypoxia inducible factors HIF1α and HIF2α. Experimental evidence suggests that HIF2α is the main regulatory factor in intestinal enterocytes [25,36]. Mice with targeted deletion of intestinal HIF2α showed an 80% reduction in DMT1 mRNA expression [25,26]. In addition, expression of Dcyt*b* [25,26] and FPN [27] is also subject to regulation by HIF2α. Furthermore, HIF2α has recently been shown to regulate expression of the ATP7A copper transporter [28]. Here, basal expression of Ctr1 mRNA was decreased in three separate models of HIF2α-knockdown namely; (1) Caco-2 cells exposed to a chemical inhibitor of HIF2α translation; (2) HIF2α-siRNA-treated HuTu 80 cells; (3) intestinal-specific HIF2α knockout mice. However, blocking HIF2α did not diminish Ctr1 mRNA in hypoxic conditions. Together these data demonstrate that HIF2α is important in determining basal expression of Ctr1 (i.e. under normoxic conditions); however, our findings also suggest that hypoxic induction of Ctr1 is independent of HIF2α. Incubation with N,N′-(2,5-dichlorosulfonyl) cystamine, which suppresses cellular HIF1α levels [29] did not alter Ctr1 mRNA expression under either normoxic or hypoxic conditions, suggesting that HIF1α does not regulate Ctr1 expression in this intestinal model.

A number of non-HIF mediated effects of hypoxia on iron metabolism have been reported; for example, in hepatoma cell lines knockdown of HIF factors does not influence hypoxic regulation of hepcidin expression [37]. A number of alternative pathways, including the activity of 2-oxoglutarate-dependent oxygenases [38], the generation of reactive oxygen species and the activation of the STAT3 signaling pathway [39] and the expression of SMAD4 [40] are all modulated by hypoxia and can directly regulate hepcidin expression. Similar pathways may also be activated by hypoxia in the intestinal epithelium and might explain the HIF2α-independent regulation of metal transporter expression.

In summary, we have shown that in addition to its well characterized regulatory effects on iron transporter expression, HIF2α also modulates the basal expression of the apical membrane copper transporter Ctr1. Our studies also suggest that non-HIF pathways play a role regulating Ctr1 expression in the response to hypoxia in the intestine. These data further reinforce the intimate relationship between iron and copper homeostasis and identify HIF2α as an important regulatory factor for iron and copper metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.05.058.

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